AD_____

Award Number: W81XWH-04-1-0209

TITLE: Notch Signaling and Schwann Cell Transformation: Development of a Model System and Application to Human MPNSTs

PRINCIPAL INVESTIGATOR: Tom Kadesch, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, PA 19104-6205

REPORT DATE: March 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
31-03-2008	4th Annual	1 MAR 2004 - 28 FEB 2008

4. TITLE AND SUBTITLE
Notch Signaling and Schwann Cell Transformation: Development of a Model System and Application to Human MPNSTs

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0209

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Tom Kadesch, Ph.D.

Email: kadesch@mail.med.upenn.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
Philadelphia, PA 19104-6205

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This is an annual report that presents data obtained during the grant's third year of funding. The grant addresses the potential role of Notch signaling in the malignant transformation of neurofibromas to MPNSTs in patients with NF1. Our previous work has shown that constitutive expression of Notch can transform rat Schwann cells and that at least on MPNST-derived human Schwann cell line (of three examined) signals via Notch. This report includes novel results pertaining to two Tasks of the Statement of Work, including our observations 1) that the Notch targets Hes5 and c-Myc alone are unable to mimic the constitutive form of Notch, NICD, to effect transformation and 2) that NICD is NOT sufficient to transform primary Schwann cells. This latter observation is in stark disagreement with our earlier results. Accordingly, we have added a new Task that will address this discrepancy and elucidate the specific pathways that NICD alters in Schwann cells.

15. SUBJECT TERMS
NF1, neurofibroma, MPNST, Notch, Schwann, transformation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

The goals of the project are to gain insights into the mechanism by which Notch (in the form of the intracellular domain, NICD) transforms rat Schwann cells, and to establish the relationship, if any, between Notch signaling and human malignant peripheral nerve sheath tumors, MPNSTs.

Notch comprises a family of transmembrane receptors whose interaction with ligand leads to proteolytic cleavages that liberate the Notch intracellular domain, NICD, from the plasma membrane. NICD then enters the nucleus where it activates transcription. Notch's role in several cancers is well established, most notably in T-ALL (T-cell acute lymphocytic leukemia) where a rare chromosomal translocation interrupts the Notch1 gene, resulting in the constitutive expression of NICD. Recent work has shown that nearly 50% of T-ALLs carry more subtle mutations in the Notch1 gene(*1*). We have shown that forced expression of NICD can transform rat Schwann cells and that one of our three MPNST cell lines expresses detectable NICD(*2*). We therefore proposed that Notch signaling may contribute to the malignant transformation of a subset of neurofibromas in NF1 patients.

Note: During the past year there have been no published studies concerning the mechanism of Schwann cell transformation that would influence the Statement of Work. *However, last year the Statement of Work was significantly altered due to unanticipated results; namely, that we were unable to repeat our original observation that NICD could transform primary Schwann cells.*

BODY

Results generated over the past year focused initially on repeating our initial observation concerning NICD's ability to "transform" Schwann cells. Our inability to do so and our early attempts to rectify the problem are provided in last year's report.

The technician working on this project was replaced year ago. Her replacement was trained and given the task of starting from the beginning: making new NICD-expressing viruses, transforming existing and new stocks of Schwann cells and evaluating the results.

This process took several months. In the end, we were able to replicate our initial observation and determined that the fault was in the particular batch of Schwann cells we had been using. Upon obtaining a fresh batch we were able to obtain, not necessarily transformation per se (see below), but efficient down-regulation of Sox10, an event seen in our initial studies.

As described in our last report, we proposed a new task:

New Task: Determine which class of oncogenes cooperates with NICD to transform Schwann cells.

Dr. Allison Lloyd and colleagues have described a general model for the transformation of rat Schwann cells(*4*). Experiments employing activated Ras and various versions of SV40 Large T antigen showed that mitogen-independent growth can be distinguished from mitogen-independent *plus* anchorage-independent growth. Both are required for Schwann cells to be tumorigenic: the former required both activation of Ras and inactivation of p53, while the latter required, in addition, the inactivation of Rb family members, most likely p107 and/or p130. Interestingly, the ability of T antigen to repress Rb family proteins could be phenocopied by mutations in the cyclin-dependent kinase inhibitor $p16^{Ink4a}$. This suggests that $p16^{Ink4a}$ mutations, while broadly stimulating CDK4 and CDK6, affect primarily the activities of p107 and p130, and not Rb. The reason for this is unknown.

We tested the hypothesis that NICD requires additional events to transform Schwann cells (as determined by anchorage independence) and that these will reflect one or a combination of those events described by Lloyd. Accordingly, we generated Schwann cells that were transduced with NICD plus either a) activated Ras, or b) SV40 Large T antigen. We anticipated that Ras plus T antigen would transform Schwann cells in the absence of NICD(*5*). According to our hypothesis, we expected NICD to fulfill the role of either activated Ras or T antigen.

An evaluation of cell growth in soft agar (a measure of anchorage independence) confirmed that a combination of Ras plus T antigen led to robust colony formation (below right), while cells containing a) Ras alone (not shown), b) T antigen alone (not shown) or c) parental vectors alone (below left) produced no colonies as expected.

pBabe + LXSN T Antigen + Ras

As reported previously, our initial experiments with NICD gave rise to modest colony

formation in soft agar (below right) while cells expressing parental vector alone did not produce any growth (below left). Note, however, that the number of colonies was greatly reduced compared to the combination of Ras plus T antigen (8 vs. >400).

MigR NICD

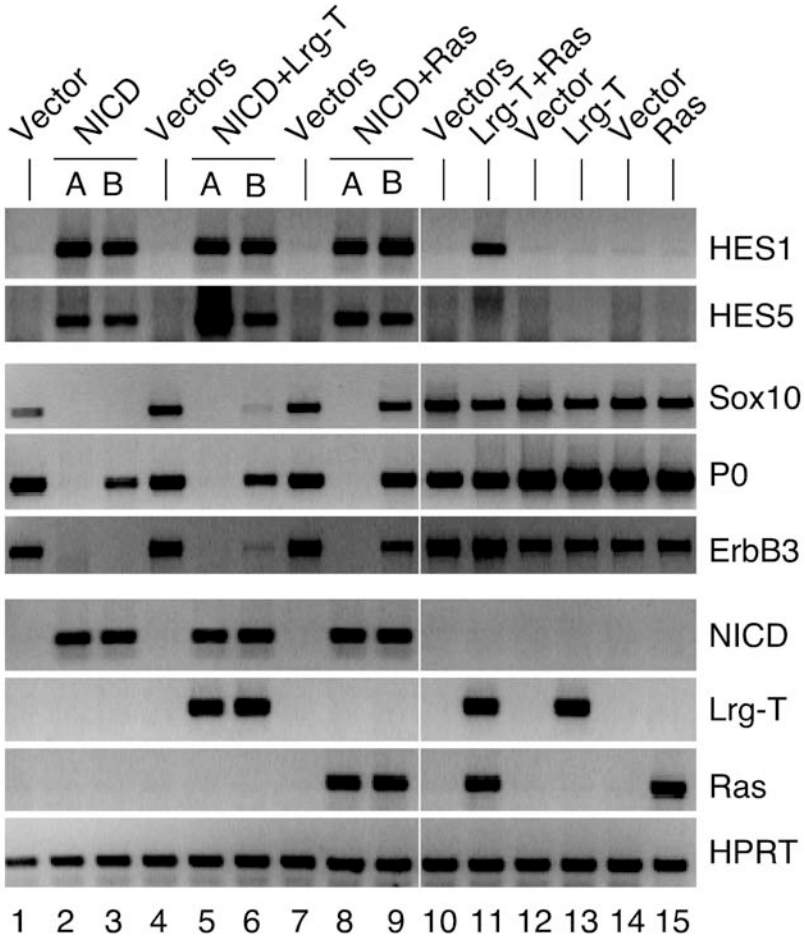
Importantly, a combination of NICD plus T antigen also gave robust growth in soft agar (below right, approx. 200 colonies), while NICD plus Ras did not increase the number of colonies over NICD alone (data not shown). *We conclude that NICD is fulfilling the role of Ras in promoting anchorage independent growth of Schwann cells.*

MigR + pBabe NICD + T Antigen

Is there a molecular signature for anchorage independence?

We had initially speculated that transformation of Schwann cells was accompanied by de-differentiation; namely, the loss of certain Schwann cell markers such as the transcription factor Sox10 and the myelin protein P0. In this case, the down-regulation of such markers by NICD would not be direct, but be secondary to the transformation process.

Given a better sense of what's required for transformation (i.e. anchorage independent growth), we have begun to explore this idea more rigorously. We have looked at gene expression by RT-PCR in Schwann cells transformed with various combinations of NICD, Ras and T-antigen. The results are shown below. Cells labeled "A" are the NICD-transduced cells we generated in our original studies. Cells labeled "B" are those generated within the past 6 months. "Lrg-T" indicates those cells transduced with a virus expressing SV40 Large T antigen. "Vectors" indicates those cells transduced with the relevant parental retroviruses.



Several conclusions can be drawn from the gene expression data shown above.

First, cell lines A and B (lanes 2 and 3) express similar levels of NICD and show similar induction of the Notch targets Hes1 and Hes 5. They also show similar down-regulation of Sox10, a likely Hes5 target. ErbB3 down-regulation is also similar in the two cell lines. However, down-regulation of P0, a Sox10 target, is much more robust in cell line A. The reason for this discrepancy is unknown.

Second, de-differentiation does not correlate with transformation. The cells capable of growing in soft agar are those transformed with combinations of Ras plus T antigen and of NICD plus T antigen (lanes 5, 6 and 11). Only those containing NICD showed down-regulation of Sox10 and ErbB3. Accordingly, it is now more likely that Sox10 and ErbB3 respond directly to Notch or to one of Notch's primary targets such as Hes1 or Hes5.

The experiments proposed for the next 6 months (the time allotted for a no-cost extension) will address a) if Sox 10 and ErbB3 are down-regulated in either Hes1- or Hes5-transduced cells (testing the aforementioned possibility) and b) if NICD, in substituting for Ras in transforming Schwann cells, induces "classic" Ras activities such as promoting MAPK phosphorylation.

KEY RESEARCH ACCOMPLISHMENTS

1. NICD cooperates with SV40 Large T antigen to stimulate growth of Schwann cells in soft agar. Thus, NICD possesses activities similar to the Ras oncogene.

2. NICD-mediated down-regulation of Sox10 and ErbB3 is not secondary to Schwann cell transformation.

REPORTABLE OUTCOMES

None.

CONCLUSIONS

We have now begun to unravel the mechanism through which NICD transforms Schwann cells and also down-regulates Sox10 and ErbB3. NICD appears to act similarly to Ras in promoting anchorage-independent growth. Transformation and Sox10/ErbB3 down-regulation are not necessarily linked.

REFERENCES

1. A. P. Weng *et al.*, *Science* 306, 269 (Oct 8, 2004).
2. Y. Li *et al.*, *Oncogene* 23, 1146 (Feb 5, 2004).
3. R. A. Woo, R. Y. Poon, *Genes Dev* 18, 1317 (Jun 1, 2004).
4. P. J. Mitchell, E. Perez-Nadales, D. S. Malcolm, A. C. Lloyd, *Mol Cell Biol* 23, 2530 (Apr, 2003).
5. A. J. Ridley, H. F. Paterson, M. Noble, H. Land, *Embo J* 7, 1635 (Jun, 1988).
6. M. C. Harrisingh *et al.*, *Embo J* 23, 3061 (Aug 4, 2004).